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(71) Applicant *(for all designated States except US)*: WISCONSIN ALUMNI RESEARCH FOUNDATION [US/US]; 614 Walnut Street, 13th Floor, P.O. Box 7365, Madison, WI 53707-7365 (US).

(72) Inventors; and

(75) Inventors/Applicants *(for US only)*: SUTHERS, Patrick, F. [US/US]; 806 Olin Avenue, Apartment 1, Madison, WI 53715 (US). CAMERON, Douglas, C. [US/US]; 3590 Rainier Lane, North Plymouth, MN 55447 (US).

(74) Agent: SEAY, Nicholas, J.; Quarles & Brady LLP, P.O. Box 2113, Madison, WI 53701-2113 (US).

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(54) Title: PRODUCTION OF 3-HYDROXYPROPIONIC ACID IN RECOMBINANT ORGANISMS

(57) Abstract: The production of 3-hydroxypropionic acid (3-HP) from glycerol in a bacterial host is described. 3-HP is a useful feedstock for the production of polymeric materials. The genetic engineering of a bacterial host with two enzymes is sufficient to enable production of 3-HP. One enzyme is a glycerol dehydratase and the other is an aldehyde dehydrogenase.

PRODUCTION OF 3-HYDROXYPROPIONIC ACID IN RECOMBINANT ORGANISMS

CROSS-REFERENCE TO RELATED APPLICATIONS

This application claims priority from U.S. Provisional Patent Application S.N. 60/151,440 filed August 30, 1999.

STATEMENT REGARDING FEDERALLY SPONSORED RESEARCH OR DEVELOPMENT

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BACKGROUND OF THE INVENTION

The technology of genetic engineering allows the transfer of genetic traits between species and permits, in particular, the transfer of enzymes from one species to others. These techniques have first reached commercialization in connection with high-value added products such as pharmaceuticals. The techniques of genetic engineering are equally applicable and cost effective when applied to genes and enzymes which can be used to make basic chemical feedstocks.

A metabolic pathway of interest exists in the bacteria *Klebsiella pneumoniae*, which has the ability to biologically produce 3 - hydroxypropionaldehyde from glycerol. Native microorganisms have the ability to produce 1,3 - propanediol from glycerol as well. Commercial interests are exploring the production of 1,3 - propanediol from glycerol or glucose, in recombinant organisms which have been engineered to express the enzymes necessary for 1,3 - propanediol production from other organisms.

3 - hydroxypropionic acid CAS registry Number [503-66-2] (abbreviated as 3-HP) is a three carbon non-chiral organic molecule. The IUPAC nomenclature name for

this molecule is propionic acid 3 - hydroxy. It is also known as 3 - hydroxypropionate, β - hydroxpropionic acid, β - hydroxypropionate, 3 - hydroxypropionic acid, 3 - hydroxypropanoate, hydracrylic acid, ethylene lactic acid, β -lactic acid and 2 - deoxyglyceric acid. Applications of 3-HP include the manufacture of absorbable prosthetic devices and surgical sutures, incorporation into beta-lactams, production of acrylic acid, formation of trifluromethylated alcohols or diols, polyhydroxyalkonates, and co-polymers with lactic acid. 3-HP for commercial use is now commonly produced by organic chemical syntheses. The 3-HP produced and sold by these methods is relatively expensive, and it would be cost prohibitive to use it for the production of monomers for polymer production. As discussed below, some organisms are known to produce 3-HP. However, there is not yet available a catalog of genes from these organisms and thus the ability to synthesize 3-HP using the enzymes natively responsible for the synthesis of that molecule in the native hosts which produce it does not now exist.

In addition to its commercial utility, 3-HP it is found in a number of biological processes, notably including many naturally occurring bio-polymers. Poly(3 - hydroxybutyrate) (PHB) is the most abundant member of the microbial polyesters which contain hydroxy monomers termed polyhydroxyalkonates (PHAs). PHB has utility as a biodegradable thermoplastic material and the material was first produced industrially in 1982.

The majority of published research on PHA's that contain 3-HP has concentrated on two bacterial sources: *Ralstonia eutropha* ("*Alcaligenes eutrophus*") and *Pseudomonas oleovorans*. Both *Ralstonia eutropha* and *Pseudomonas oleovorans* are able to grow on a nitrogen free media containing 3 - hydroxy - propionic acid, 1,5 - pentanediol or 1,7 - heptanediol. When 3-HP is the major hydroxy-acid added to the growth media, poly(3 - hydroxybutyrate - co - 3 - hydroxypropionic acid) is formed containing 7 mol % 3 - hydroxypropionic acid. These cells also store 3 mol %, 3 - hydroxypropionic acid poly(3 - butyrate - co - 3 - hydroxypropionic acid).

Recombinant systems have been used to create PHAs. An *E. coli* strain engineered to express PHA synthase from either *Ralstonia eutropha* or *Zoolgoea ramigera* produced poly(3 - hydroxypropionic acid) when feed 1,3 - propanediol.

Skraly, F. A. "Polyhydroxyalkanoates Produced by Recombinant *E. coli.*" Poster at Engineering Foundation Conference: Metabolic Engineering II, 1998. An *E. coli* strain that expressed PHA synthase (MBX820), when provided with the genes encoding glycerol dehydratase and 1,3 - propanediol dehydratase from *K. pneumonia* , and 4 - hydroxybutyral- CoA transferase from *Clostridium kluyveri,* synthesized PHB from glucose.

Glycerol dehydratase, found in the bacterial pathway for the conversion of glycerol to 1,3 - propanediol, catalyzes the conversion of glycerol to 3 - hydroxypropionaldehyde and water. This enzyme has been found in a number of bacteria including strains of *Citrobacter, Klebsiella, Lactobacillus, Entrobacter* and *Clostridium*. In the 1,3 - propanediol pathway a second enzyme 1,3 - propanediol oxido-reductase (EC 1.1.202) reduces 3 - hydroxypropanaldehyde to 1,3 - propanediol in a NADH dependant reaction. The pathway for the conversion of glycerol to 1,3 - propanediol has been expressed in *E. coli*. Tong et al., Applied and Environmental Microbiology 57 (12) 3541-3546. The genes responsible for the production of 1,3 - propanediol were cloned from the *dha* regulon of *Klebsiella pneumoniae*. Glycerol is transported into the cell by the glycerol facilitator, and then converted into 3 - hydroxy - propionaldehyde by a coenzyme B_{12}- dependent dehydratase. *E. coli* lacks a native *dha* regulon, consequently *E. coli* cannot grow anaerobically on glycerol without an exogenous electron acceptor such as nitrate or fumarate.

Aldehyde dehydrogenases are enzymes that catalyze the oxidation of aldehydes to carboxylic acids. The genes encoding non-specific aldehyde dehydrogenases have been identified in a wide variety of organisms e.g.; *ALDH2* from *Homo sapiens*, *ALD4* from *Saccharomyces cerevisiae*, and from *E. coli* both *aldA* and *aldB,* to name a few. These enzymes are classified by co-factor usage, most require either AND^+, or $NADP^+$ and some will use either co-factor. The genes singled out for mention here are able to act on a number of different aldehydes and it likely that they may be able to oxidize 3 - hydroxy - propionaldehyde to 3 - hydroxypropionic acid.

BRIEF SUMMARY OF THE INVENTION

The present invention is intended to permit the creation of a recombinant microbial host which is capable of synthesizing 3-HP from a starting material of glycerol or glucose. The glycerol or glucose is converted to 3 - hydroxypropionicaldehyde (abbreviated as 3-HPA) which is then converted to 3-HP. This process requires the so-called *dhaB* gene from *Klebsiella pneumoniae* which encodes the enzyme glycerol dehydratase any one of four different aldehyde dehydrogenase genes to convert 3-HPA to 3-HP. The four aldehyde dehydrogenase genes used were *aldA* from the bacterium *E. coli*, *ALDH2* from humans, *ALD4* from the yeast *Saccharomyces cerevisiae*, and *aldB* from *E. coli*. The yeast gene appeared to give the best results.

It is an object of the present invention to provide a genetic construct which encodes glycerol dehydratase and aldehyde dehydrogenase enzymes necessary for the production of 3 - hydroxypropionic acid from glycerol.

It is also an object of the present invention to provide a method for the production of 3 - hydroxypropionic acid from glycerol.

Other features and advantages of the invention will be apparent from the following description of the preferred embodiment thereof and from the claims.

BRIEF DESCRIPTION OF THE SEVERAL VIEWS OF THE DRAWINGS

Not applicable.

DETAILED DESCRIPTION OF THE INVENTION

It is disclosed here that it is possible to introduce into a bacterial host genes encoding two enzymes and thus confer upon that host the ability to produce 3-HP from glycerol. The two necessary enzymes are glycerol dehydratase and aldehyde dehydrogenase. It is here reported that the two enzymes are both necessary and sufficient to enable a strain of a suitable host, such as a competent *E. coli* strain, to make 3-HP from glycerol. An exemplary gene encoding a glycerol dehydratase is known, the *dhaB* gene from *Klebsiella pneumoniae*, sequenced and rendered convenient to use. Several exemplary aldehyde dehydrogenases are known, and their sequences are

presented here. From this information, it becomes practical to confer upon a bacterial host the ability to convert glycerol into 3-HP in a commercially reasonable manner.

It was not apparent before the completion of the work described here that these two diverse enzymes could be produced in a common host to produce the ability to make 3-HP. There are many known aldehyde dehydrogenase enzymes and genes, and the enzymes are known to have varying substrate specificities and efficiencies. There was not evidence, prior to the work described here, that the aldehyde dehydrogenase enzyme would work on the 3-hydroxypropionicaldehyde (3-HPA) substrate to create 3-HP. Without that knowledge, there was no data from which to predict the effectiveness of the 3-HP production studies described below. An additional uncertainty arises from the fact that the intermediate aldehyde, 3-HPA, is toxic to many bacterial host and thus the survival of the host is dependent upon the relative rates of enzymatic production and conversion of the aldehyde intermediate to non-toxic 3-HP.

A difficulty in the realization of the production of 3-HP desired here is that ribosome binding sites from non-native hosts are often ineffectual and lead to poor protein production and that many non-native promoters are often poorly transcribed and a bar to high protein expression. However, the inventors also recognized that a non-native promoter that is known to be very active and is inducible by the addition of a small molecule unrelated to the pathway being expressed is often a very efficient way to express and regulate the levels of enzymes expressed in hosts such as *E. coli*. To achieve high levels of regulated gene expression plasmids were constructed which placed the expression of all exogenous genes necessary for the production of 3 - hydroxypropionic acid from glycerol under the regulation of the *trc* promoter. The *trc* promoter, is efficient, not native to *E. coli*, and inducible by the addition of IPTG.

The present specification describes a genetic construct for use in the production of 3 - hydroxypropionic acid from glycerol. The genetic construct includes exemplary DNA sequences coding for the expression of a glycerol dehydratase and a DNA sequence coding for aldehyde dehydrogenase. The set of exemplary sequences necessary for the expression of glycerol dehydratase is collectively referred to as "*dhaB*". The set of sequences necessary for the expression of aldehyde dehydrogenase includes any one of four different genes which proved efficacious. The individual

aldehyde dehydrogenase sequences referred to individually as *ALDH4*, *ALD2*, *aldA* and *aldB*.

Producing 3 - hydroxypropionic acid in a foreign host

In the work described below, the enzymes necessary for the production of 3 - hydroxypropionic acid from glycerol in *E. coli* were expressed under the regulation of the *trc* promoter, a non-native promoter inducible by the addition of IPTG. The glycerol dehydratase was encoded by the *dhaB* gene from *Klebsiella pneumoniae*, the aldehyde dehydrogenases used was any one of four different genes (*ALDH2* from *Homo sapiens*, *ALD4* from *S. cerevisiae*, *aldB* from *E. coli* or *aldA* from *E. coli*). Expression of these genes coding for glycerol dehydratase and any one of the genes encoding an aldehyde dehydrogenases was sufficient to enable the construct to produce 3-HP when the fermentation media was supplemented with glycerol. In all of these constructs, the *dhaB* gene was downstream from the gene encoding the aldehyde dehydrogenase used, and expression of both genes was regulated by the *trc* promoter. This order, however, is not required and the order of the gens on a construct and the use of multiple constructs is possible.

In a minimal genetic construct made based on the data presented here, the only genetic elements present that would be necessary are the structural genes *dhaB* and an aldehyde dehydrogenase gene encoding a protein that efficiently catalyzes the oxidation of 3-hydroxypropionaldehyde to 3-hydroxypropionic acid, and non-native promoter sequences specifically selected to give the type of inducible control most appropriate for the context of the process in which the construct is to be used. Extraneous pieces of DNA, whether retained in the construct or added from other DNA sequences, would not necessarily be detrimental to effective 3-HP synthesis by the host organism, but would not be needed. Each sequence to be translated would necessarily be preceded by a ribosome binding site, functional in the selected host so that the messenger RNA(s) coding for the proteins of interest could be translated by ribosomes. Terminator sequences immediately downstream of each translated unit would also be necessary in some organisms, particularly in eukaryotes. The construct could be part of an autonomously replicating sequence, such as a plasmid or phage vector, or could be

integrated into the genome of the host.

The structural genes and appropriate promoter(s) could be isolated by the use of restriction enzymes, by the polymerase chain reaction (PCR), by chemical synthesis of the appropriate oligonucleotides, or by other methods apparent to those skilled in the art or molecular biology. The promoter(s) would be derived from genomic DNA of other organism or from artificial genetic constructs containing promoters. Appropriate promoter fragments would be ligated into the construct upstream of the structural genes in any one of several possible arrangements.

The aldehyde dehydrogenase expressed would have: high specific activity towards 3-hydroxypropionaldehyde; be very stable in the host it is expressed in; be readily over expressed in the selected host; not be inhibited by either the substrates necessary for the reaction or the products formed by the reaction; be fully active under the fermentation conditions most favorable for the production of 3 - hydroxypropionic acid and be able to use either NAD^+ or $NADP^+$.

One possible arrangement is the true operon, where one promoter is used to direct transcription in one direction of all necessary Open Reading Frames (ORFs). The entire message is then contained in one messenger RNA. The advantages of the operon are that it is relatively easy to construct, since only one promoter is needed; that is it is relatively simple to replace the promoter with another promoter if that would be desirable later; and that it assures that the two genes are under the same regulation. The main disadvantage of the operon scheme is that the levels of the expression of the two genes cannot be varied independently. If it is found that the genes, for optimal 3 - hydroxypropionic acid synthesis, should be expressed at different levels, the operon in most cases cannot be used to realize this.

Another possible arrangement is the multiple-promoter scheme. Two or more promoters, with the same or distinct regulatory behavior, could be used to direct transcription of the genes. For example, one promoter could be used to direct transcription of *dhaB* and one to direct transcription of the gene encoding the appropriate aldehyde dehydrogenases. Because the genes theoretically can be transcribed and translated separately, a great number of combinations of multiple promoters is possible. Additionally, it would be most desirable to prevent the promoters

from interfering with one another. This could be achieved either by placing two promoters into the construct such that they direct transcription in opposite directions, or by inserting transcriptional terminator sequences downstream of each separately transcribed unit. The main advantage of the multiple-promoter construct is that it permits independent regulation of as many distinct units as desired, which could be important. The disadvantages are that it would be more difficult to construct; more difficult to amend later; and more difficult to effectively regulate, since multiple changes in fermentation conditions would need to be introduced and might render the performance of the fermentation somewhat less predicable.

In any construct, the promoter sequence(s) used should be functional in the selected host organism and preferably provide sufficient transcription of the genes comprising the glycerol to 3 - hydroxypropionic acid pathway to enable the construct to be adequately active in that host. The promoter sequence(s) used would also effect regulation of transcription of the genes enabling the glycerol to 3-HP pathway to be adequately active under the fermentation conditions employed for 3-HP production, and preferably they would be inducible, such that expression of the genes could be modulated by the inclusion in, or exclusion from, the fermentation of a certain agents or conditions.

A plausible example of the use of such a construct follows: one promoter, which induced by the addition of an inexpensive chemical (the inducer) to the medium, could control transcription of both the *dhaB* gene and the gene encoding the appropriate aldehyde dehydrogenase. The cells would be permitted to grow in the absence of the inducer until they accumulated to a predetermined level. The inducer would then be added to the fermentation and nutritional changes commensurate with the altered metabolism would be made to the medium as well. The cells would then be permitted to utilize the substrate(s) provided for 3-HP production (and additional biomass production if desired). After the cells could no longer use substrate to produce 3-HP, the fermentation would be stopped and the 3-HP recovered.

Genetic Sequences

To express glycerol dehydratase and a suitable aldehyde dehydrogenase, the two

enzymes necessary for the production of 3 - hydroxypropionic acid from glycerol, it is required that the DNA sequences containing the glycerol dehydratase and aldehyde dehydrogenase coding sequences be combined with at least a promoter sequence (preferably a non-native promoter although some native promoter activity may be present). An exemplary method of construction is described in the example below. To ensure that the present specification is enabling, the full sequences of the coding regions of genes for these enzymes is presented here.

Sequences 1, 3, 5 and 7 present different native genomic sequences for genes encoding aldehyde dehydrogenases.

SEQ ID NO:1 contains the full native DNA sequence encoding the *ALD4* enzyme from *Saccharomyces cerevisiae*. The amino acid sequence of the protein is presented as SEQ ID NO:2.

SEQ ID NO:3 includes the DNA sequence for the human *ALDH2* gene, again including the full protein coding region. The amino acid sequence for this human alcohol dehydrogenase is presented in SEQ ID NO:4.

SEQ ID NO:5 and 7 respectively present the full coding sequences from the E. coli genes aldA and aldB, both of which encode alcohol dehydrogenases. The amino acid sequences for the proteins encoded by the genes are presented in SEQ ID NO: 6 and 8 respectively.

SEQ ID NO:9 contains the native genomic DNA sequence for the *dhaB* gene from the *dha* regulon of *Klebisiella pneumoniae*. The coding sequences for this complex regulon produces five polypeptides, which are presented as SEQ ID NOS:10 through 13, which together provide the activity of the glycerol dehydratase enzyme.

Each of these coding sequences can be used to make genetic constructs for the expression of the appropriate enzymes in a heterologous hosts. In making genetic constructs for expression of the genes in such hosts, it is contemplated that heterologous promoters will be joined to the coding sequences for the enzymes, but all that it required is that the promoters be effective for the hosts in which the genes are to be expressed. It is also contemplated and envisioned that significant variations in DNA sequence are possible from the native DNA coding sequences presented here. As is well known in the art, due to the degeneracy of the genetic code, many different DNA sequences can

encode the expression of the same protein. So, when this document uses language specifying a DNA sequence encoding a protein, it is intended to encompass any DNA sequence which can be used to express that protein even if different from the genomic sequences presented here. It is also contemplated that conservative changes in the amino acid sequences of the proteins specified here can be made without departing from the present invention. In particular, deletions, additions and substitutions of one or more amino acids in a protein sequence can almost always be made without changing protein functionality. When the name of a protein is sued here, it is intended to be equally applicable to both such minor changes in amino acid sequence and to allelic variations in native protein sequence as occurs within the species named as well as other closely related species.

It is possible that many of the above DNA sequences could be truncated and still express a protein that has the same enzymatic properties. One skilled in the art of molecular biology would appreciate that minor deletions, additions and mutations may not change the attributes of the designated base pair sequences; many of the nucleotide of the designated base pair sequences are probably not essential for their unique function. To determine whether or not an altered sequence or sequences has sufficient homology with the designated base pairs to function identically, one would simply create the candidate mutation, deletion or alteration and create a gene construct including the altered sequence together with promoter and termination sequences. This gene construct could be tested as, described below, for the production of 3-HP from glycerol.

Certain DNA primers were used to isolate or clone the genomic DNA sequences used in the experiments described below. While the sequence information presented here is sufficient to enable the construction of expression plasmids incorporating the genes identified here, in order to redundantly enable the use of these genes, primers which may be used to isolated the genes from their native hosts are described below.

The primers aldA_L (SEQ ID NO:14), and aldA_R (SEQ ID NO:15), were used to amplify the 1513 bp *ald*A fragment from genomic *E. coli* DNA (strain MG1655, a gift from the Genetic Stock Center, New Haven, CT). The gel purified PCR fragment containing a DNA sequence coding for the expression of aldehyde dehydrogenase was

inserted into *Nco*I-*Xho*I site of pSE380 (Invitrogen, San Diego, CA) to give pPFS3. The resulting plasmid contained *ald*A under the control of the *trc* promoter. This construct allowed for high-level expression of the *ald*A gene from *E. coli* under regulation of the *trc* promoter. Unless indicated otherwise all molecular biology and plasmid constructions were done in *E. coli* AG1 (Stratagene, La Jolla, CA).

The primers aldB_L (SEQ ID NO:20) and aldB_R (SEQ ID NO:21), were used to amplify the 1574 bp *ald*B fragment from genomic *E. coli* DNA (strain MG1655). The resulting PCR converted the TGA stop codon into a TAA stop codon. The gel-purified PCR fragment containing the DNA sequence sufficiently coding for the expression of aldehyde dehydrogenase was inserted into the *Kpn*I-*Sac*I site of pSE380 to give pPFS12.

The primers ALD4_L (SEQ ID NO : 16), and ALD4_R (SEQ ID NO : 17), were used to amplify the 1595 bp ALD4 fragment from *S. cerevisiae* DNA (strain YPH500). The gel-purified fragment containing a DNA sequence coding for the expression of aldehyde dehydrogenase was inserted into the *Kpn*I-*Sac*I site of pPFS3 to give pPFS8. The resulting plasmid contained mature *ALD4* under control of the *trc* promoter.

The primers ALDH2_L (SEQ ID NO:18), and ALDH2_R (SEQ ID NO:19), were used to amplify the 1541 bp ALDH2 fragment from pT7-7::ALDH2, a gift from H. Weiner (Purdue University, West Lafayette, IN). The gel purified PCR fragment containing a DNA sequence sufficiently homologous to base pairs 22 to 1524, inclusive of SEQ ID NO : 3 so as to code for the expression of aldehyde dehydrogenase was inserted in to the *Kpn*I-*Sac*I site of pSE380 to give pPFS7. This sequence was moved from pPFS7 into the *Kpn*I-*Sac*I site of pPFS3 to give pPFS9. The resulting plasmid contained mature ALDH2 under the control of the *trc* promoter.

The primers pTRC_L (SEQ ID NO:22), and pTRC_R)SEQ ID NO:23), were used to amplify the 540 bp fragment from pSE380. The gel purified PCR fragment was inserted into the *Hpa*I-*Kpn*I site of pPFS3 to give pPFS13. The resulting plasmid deleted the "native" ribosome binding site of pSE380 and a *Nco*I site (which contained an extraneous ATG start codon upstream of the cloned genes). The *Kpn*I-*Sac*I fragments of pPFS8, pPFS9, and pPSF12 were inserted into the *Kpn*I-*Sac*I site of pPFS13 to give pPFS14, pPFS15, and pPFS16, respectively.

Assay for production of 3-HP

The efficacy of changes made as contemplated herein can be checked by the following tests. To test for the production of 3-HP, fermentation products can be quantified with a Waters Alliance Integrity HPLC system (Milford, MA) equipped with a refractive index detector, a photodiode array detector, and an Aminex HPX-87H (Bio-Rad, Hercules, CA) organic acids column. The mobile phase should be 0.01 N sulfuric acid solution (pH 2.0) at a flow rate of 0.5 mL/min. The column temperature should be set to 40°C. Compounds can be identified by determining if they co-elute with authentic standards. Prior to analysis, all samples should be filtered through 0.45 μM pore size membrane. (Gelman Sciences, Ann Arbor, MI). The fractions of the fermentation products collected using HPLC should be analyzed on a Varian Star 3400 CX, gas - chromatograph coupled to a Varian Saturn 3 mass spectrometer (GC-MS) (Walnut Creek, CA).

Assay for enzyme activity.

Aldehyde dehydrogenase activity can be determined by measuring the reduction of β-NAD^+ at 25°C with 3 - hydroxypropionaldehyde as a substrate. All buffers should contain 1 mM ethylenediaminetetraacetic acid (EDTA), 0.1 mM Pefabloc SC (Boehringer Mannheim, Indianapolis, IN) and 1 mM Tris (carboxyethyl) phosphine hydrochloride (TCEP-HCL). For ALD4, the solution should contain 100 mM Tris HCL Buffer (pH 8.0), 100 mM KCl. For ALDH2 the solution should contained 100 mM sodium pyrophosphate (pH 9.0). For AldA and AldB, the solution should contain 20 mM sodium glycine (pH 9.5). A total of 3.0 mL of buffer should be added to quartz cuvettes and allowed to equilibrate to assay temperature. From 5 to 20 μL of cell extract should be added and background activity recorded after the addition of β-NAD^+ to a final concentration of 0.67 mM. The reaction should be started by the addition of substrate (either acetaldehyde, propionaldehyde, or 3 - hydroxypropionaldehyde) to a final concentration of 2 mM. Assay mixtures should be stirred with micro-stirrers during the assays.

For aldehyde dehydrogenase activity assays, one unit is defined as the reduction

of 1.0 μM of β-AND^+ per minute at 25° C. These reactions can be monitored by following the change in absorbence at 340 nm (A_{340}) at 25°C on a Varain Carry-1 Bio spectrophotometer (Sugar Land, TX). Total protein concentrations in the cell extracts can be determined using the Bradford assay method (Bio-Rad, Hercules, CA) with bovine serum albumin as the standard.

EXAMPLES

Plasmid constructions.

Klebsiella pneumoniae expresses glycerol dehydratase, an enzyme that catalyzes the conversion of glycerol to 3 - hydroxypropionaldehyde, (*dhaB*) and 1,3 - propanediol oxidoreductase an enzyme that catalyzes the conversion of 3 - hydroxypropionaldehyde to 1,3 - propanediol respectively (the gene product from *dhaT*). A plasmid encoding these two genes was created and expressed in *E. coli* (plasmid pTC53). The *dhaT* gene was deleted from pTC53 to create pMH34. The resulting plasmid still contained the DNA sequence complementary to base pairs 330 to 2153 inclusion of SEQ ID NO : 9, the complement of base pairs 2166 to 2591, inclusive, of SEQ ID NO : 9, and the complement of base pairs 3191 to 4858, inclusive, of SEQ ID NO : 9, so as to code for the expression of glycerol dehydratase. The fragment of DNA encoding these sequences was excised from pMH34 by cutting it with *SalI-XbaI, and* the resulting fragment was gel purified (the purified fragment was gift from M. Hoffman of the University of Wisconsin - Madison). This DNA fragment was inserted into the *SalI-XbaI* site of pPFS13 to give pPFS17.

The resulting plasmid contained both the *aldA* and *dhaB* genes under the control of the *trc* promoter. Similarity, the gel-purified *SalI-XbaI* fragment from pMH34 was inserted into the *SalI-XbaI* sites of pPFS14, pPFS15, and pPFS16 to give pPFS18, pPFS19, and pPFS20, respectively. These plasmids contained *ALD4*, *ALDH2*, and *aldB*, respectively, as well as *dhaB* under the control of the *trc* promoter; in all of the constructs the *dhaB* gene were downstream of the gene encoding the aldehyde dehydrogenase.

Expression in *E. coli.*

The efficacy of *E. coli* as a platform for the production of 3-HP from growth on glucose has been examined using a mathematical model developed for this purpose. The model was executed in two different ways assuming the conversion of one mole of glucose under either anaerobic or aerobic conditions either directly to 3-HP or to the production of 3-HP and ATP. The optimum yield under anaerobic conditions is 1 mole of 3-HP and 1 mole of lactate. The more realistic yield under anaerobic conditions is 0.5 moles of 3-HP, 1.5 moles of lactate and 1 mole of ATP. The optimum yield under aerobic conditions is 1.9 moles of 3-HP and 0.3 moles of CO_2. The realistic yield under aerobic conditions is 1.85 moles of 3-HP, 0.35 moles of CO_2 and 1 mole of ATP.

The effect of 3-HP concentration on *E. coli* strain MG1655 growth was measured. Cells were grown on standard media with and without the addition of up to 80g/L of 3-HP. The best fit of these data demonstrated that 3-HP was only 1.4 times as inhibitory as lactic acid on the growth of *E. coli.* It is possible to economically produce lactic acid using *E. coli*, since 3-HP is only 1.4 times more inhibitory than lactic acid, it should be possible to use *E. coli* as a host for the commercial production of 3-HP.

Media and growth conditions

The standard media contained the following per liter: 6 g Na_2HPO_4, 3 g KH_2PO_4, 1 g NH_4Cl, 0.5 g NaCl, 3 mg $CaCl_2$, 5 g yeast extract (Difco Laboratories, Detroit, MI) and 2 mM $MgSO_4$. When necessary to retain plasmids ampicillin (100 mg/mL) was added to the media. Isopropyl-β-thiogalactopyranoside (IPTG) was added in varying amounts to induce gene expression. All fermentations were carried out in an incubator-shaker at 37 C and 200 rpm. Anaerobic fermentations were carried out in 500-mL anaerobic flasks with 300 mL of working volume. Inocula for fermentations were grown overnight in Luria-Bertani medium supplemented with ampicillin is necessary. The 300-mL fermentations were inoculated with 1.5 mL of the overnight culture. For enzyme assays, fermentations were incubated for 24 hours.

Over expression of aldehyde dehydrogenase in *E. coli.*

Cells were harvested by centrifugation at 3000 x g for 10 minutes at 4°C with a

Beckman (Fullerton, CA) model J2-21 centrifuge. Cell pellets were washed twice in 100 mM potassium phosphate buffer at pH 7.2 and re-suspended in appropriate assay resuspension buffer equal to 5 x of the volume of the wet cell mass. The cells were homogenized using a French pressure cell. The homogenate was centrifuged at 40000 x g for 30 minutes. The supernatant was dialyzed against the appropriate resuspension buffer using 10000 molecular weight cut-off pleated dialysis tubing (Pierce, Rockford, IL) at 4°C. Dialysis buffer was changed after 2 hours, and 4 hours, and dialysis was stopped after being allowed to proceed overnight.

E. coli AG1 cells transfected with the plasmids constructed to express the *aldA*, *ALD4*, *ALDH2*, or *aldB* genes were grown in 500-mL anaerobic flasks. Twelve hours after the fermentations were inoculated IPTG was added to induce enzyme expression. The cells were allowed to grow for an additional 12 hours then harvested and lysed as discussed above. The soluble fraction of the lysate was assayed for aldehyde dehydrogenase activity using the substrate 3-hydroxypropionicaldehyde in the buffer appropriate for the particular enzyme expressed The plasmid, aldehyde dehydrogenase expressed and specific activity measured (U/mg of protein) were as follows: pPFS13, *aldA*, 0.2; pPFS14, *ALD4*, 0.5, pPFS15, *ALDH2*, 0.3; and pPFS16, *aldB*. 0.1. The control, *E. coli* strain AG1 harboring plasmid pSE380, encoded no exogenous aldehyde dehydrogenase activity and it had no detectable activity with 3-HP as substrate. It is clear from the activity assays that all four aldehyde dehydrogenases were expressed in *E. coli*. The aldehyde dehydrogenase cloned from *Saccharomyces cerevisiae (ADH4)* had the highest activity when 3-hydroxypropionaldehyde was used as the substrate (0.5 units/mg of protein).

E. coli cells transformed with plasmids expressing: aldehyde dehydrogenase; both aldehyde dehydrogenase and glycerol dehydratase, or neither gene; were grown and assayed for their ability to produce 3-HP from glycerol. The cells were grown on standard media supplemented with 6 μM of Coenzyme B_{12}, under anaerobic conditions in the absence of light (to protect the integrity of the Coenzyme B_{12} necessary for DhaB activity). After 12 hours, IPTG was added to induce expression of the genes under the *trc* promoter at the same time 5g/L of glycerol was added. After 12 more hours of anaerobic fermentation the fermentation broth was assayed for 3 - HP by HPLC and GC,

the plasmid, aldehyde dehydrogenase gene expressed and g/L of 3- HP measured were as follows: pSF17, *aldA*, 0.031; pPSF18 *ALD4*, 0.173; and pPSF19, *ALDH2*, 0.061. Cells expressing *dhaB* but no exogenous aldehyde dehydrogenase genes (plasmid pMH34) produced 0.015 g/L of 3 - HP. Cells expressing *aldA*, *ALD4*, *ALDH2* or *aldB* but not *dhaB* (plasmids pPFS13, pPFS14, pPFS15, pPFS16, respectively) all produced less then 0.005 g/L of 3-HP when the media the cells were growing in was supplemented with 2.5g/L of 3-hydroxypropionaldehyde.

Other Hosts and Promoters

Applications of the 3 - hydroxypropionic acid pathway such as the genetic constructs of the present invention can easily be expressed in other organisms. The required genes would need to be placed under control of an appropriate promoter or promoters. Some organism such as yeasts may require transcription terminators to be placed after each transcribed unit. The knowledge of the present intention makes such amendments possible. Such a genetic construct would need to be part of a vector that could either replicate in the new host or integrate into the chromosome of the new host. Many such vectors are commercially available for expression in gram-negative and gram-positive bacteria, yeast, mammalian cells, insect cell, plant, etc. For example, to express the 3-hydroxypropionic acid pathway in *Rhodobacter capsulatus*, one could obtain vector pNH2 from the American Type Culture Collection (ATTC). This is a shuttle vector for use in *R. capsulatus* and *E. coli*. Organisms such as *Saccharomyces cerevisiae* which can convert glucose to glycerol could be used as a host, such a construct would enable the production of 3 - HP directly from glucose. Additionally, other substrates such as xylan could also be used given the selection of an appropriate host.

Stochiometric analysis shows that best stochiometric yield of 3-HP production in *E. coli* calculated on the basis of glucose consumed is obtained under aerobic conditions. Under aerobic condition CO_2 is the only carbon-containing co-product, in particular the generation of lactic acid which occurs under anaerobic conditions is avoided. Production of 3-HP under these conditions could result in a more economical recovery of 3-HP from the fermentation broth.

Alternatively, the *dhaB* gene and a gene encoding the appropriate aldehyde dehydrogenase could be cloned into the multiple cloning site of this vector in *E. coli* to facilitate construction, and then transformed into *R. capsulatus*. The *R. capsulatus* nifH promoter, provided on the plasmid, could be used to direct the transcription in *R. capsulatus* of the genes placed into pNF2 in series with one promoter, or with two copies of the nifH promoter. Expression of the genes in other organisms would require a procedure analogous to that presented here.

Alternative Aldehyde Dehydrogenases and Glycerol Dehydratases

Applications of the pathway for the production of 3-hydroxypropionic acid from glycerol can be made using other suitable aldehyde dehydrogenases. To be functional in this pathway an aldehyde dehydrogenase needs to be stable, readily expressed in the host of choice and have high enough activity towards 3-hydroxypropionaldehyde to enable it to make 3-HP. The knowledge of the present invention makes such amendments possible. A program of directed evolution could be undertaken to select for suitable aldehyde dehydrogenases or they could be recovered from native sources, the genes encoding these enzymes in conjunction with a gene encoding an appropriate glycerol dehydratase activity, would then be made part of any of the constructs envisioned here to produce 3 - hydroxypropionic acid from glycerol.

A similar program of enzyme improvement including for example directed evolution could be carried out using the *dhaB* gene from *Klebsiella pneumoniae* as a starting point to obtain other variants of glycerol dehydratase that are superior in efficiency and stability to the form used in this invention. Alternatively, enzymes which catalyzes the same reaction may be isolated from others organisms and used in place of the *Klebsiella pneumoniae* glycerol dehydratase. Such enzymes may be especially useful in alternative hosts wherein they may be more readily expressed, be more stable and more efficient under the fermentation conditions best suited to the growth of the construct and the production and recovery of 3-HP.

CLAIM OR CLAIMS

I/WE CLAIM:

1. A method for producing 3-hydroxypropionic acid comprising the steps of providing in a fermenter a recombinant microorganism which expresses genes for non-native enzymes which are capable of catalyzing the production of 3-hydroxypropionic acid from glycerol;

providing a source of glycerol or glucose for the recombinant microorganism, and

fermenting the microorganism under conditions which result in the accumulation of 3-hydroxypropionic acid.

2. A method for producing 3-hydroxypropionic acid comprising the steps of providing in a fermenter a recombinant microorganism which carries genetic constructions for the expression of a glycerol dehydratase and an aldehyde dehydrogenase which are capable of catalyzing the production of 3-hydroxypropionic acid from glycerol;

providing a source of glycerol or glucose for the recombinant microorganism, and

fermenting the microorganism under conditions which result in the accumulation of 3-hydroxypropionic acid.

3. A method for producing 3-hydroxypropionic acid comprising the steps of

providing in a fermenter a recombinant microorganism which carries a genetic construct which expresses the *dhaB* gene from *Klebsiella pneumoniae* and a gene for an aldehyde dehydrogenase, which are capable of catalyzing the production of 3-hydroxypropionic acid from glycerol;

providing a source of glycerol or glucose for the recombinant microorganism, and

fermenting the microorganism under conditions which result in the accumulation of 3-hydroxypropionic acid.

4. The method of claim 3 wherein the gene for the aldehyde dehydrogenase is selected from the group consisting of *ALDH4*, *ALD2*, *aldA* and *aldB*.

5. The method of claim 3 wherein the aldehyde dehydrogenase is selected from the group consisting of SEQ ID NO:2, SEQ ID NO:4, SEQ ID NO:6 and SEQ ID NO:8.

6. A recombinant *E. coli* host comprising in its inheritable genetic materials foreign genes encoding a glycerol dehydratase and an aldehyde dehydrogenase, such that the host is capable of producing 3-hydroxypropionic acid from glycerol.

7. A recombinant *E. coli* host comprising in its inheritable genetic materials the *dhaB* gene from *Klebsiella pheumoniae* and the *ald4* gene from *Saccharomycetes cervisiae*, such that the host is capable of producing 3-hydroxypropionic from glycerol.

8. A bacterial host comprising in its inheritable genetic material a genetic construction encoding for the expression of a glycerol dehydratase enzyme and an aldehyde dehydrogenase enzyme, such that the bacterial host is capable of converting glycerol to 3-hydroxypropionic acid.

9. The bacterial host of claim 8 wherein the glycerol dehydratase from *Klebsiella pneumoniae*.

10. The bacterial host of claim 8 wherein the gene encoding the glycerol dehydratase is the *dhaB* gene from *Klebsiella pneumoniae*.

11. The bacterial host of claim 8 wherein the aldehyde dehydrogenase is selected from the group consisting of SEQ ID NO:2, SEQ ID NO:4, SEQ ID NO:6 and SEQ ID NO:8.

12. The bacterial host of claim 8 wherein the gene for the aldehyde dehydrogenase is selected from the group consisting of *ALDH4*, *ALD2*, *aldA* and *aldB*.

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Cameron, Douglas C.

<120> Production of 3-Hydroxypropionic Acid in Recombinant Organisms

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355 360 365

Leu Cys Gly Gly Gly Ile Ala Ala Asp Arg Gly Tyr Phe Ile Gln Pro
370 375 380

Thr Val Phe Gly Asp Val Gln Asp Gly Met Thr Ile Ala Lys Glu Glu
385 390 395 400

Ile Phe Gly Pro Val Met Gln Ile Leu Lys Phe Lys Thr Ile Glu Glu
405 410 415

Val Val Gly Arg Ala Asn Asn Ser Thr Tyr Gly Leu Ala Ala Ala Val
420 425 430

Phe Thr Lys Asp Leu Asp Lys Ala Asn Tyr Leu Ser Gln Ala Leu Gln
435 440 445

Ala Gly Thr Val Trp Val Asn Cys Tyr Asp Val Phe Gly Ala Gln Ser
450 455 460

Pro Phe Gly Gly Tyr Lys Met Ser Gly Ser Gly Arg Glu Leu Gly Glu
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Tyr Gly Leu Gln Ala Tyr Thr Glu Val Lys Thr Val Thr Val Lys Val
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Pro Gln Lys Asn
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Met Ser Val Pro Val Gln
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cat cct atg tat atc gat gga cag ttt gtt acc tgg cgt gga gac gca 102
His Pro Met Tyr Ile Asp Gly Gln Phe Val Thr Trp Arg Gly Asp Ala
10 15 20

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tgg att gat gtg gta aac cct gct aca gag gct gtc att tcc cgc ata   150
Trp Ile Asp Val Val Asn Pro Ala Thr Glu Ala Val Ile Ser Arg Ile
         25                  30                  35

ccc gat ggt cag gcc gag gat gcc cgt aag gca atc gat gca gca gaa   198
Pro Asp Gly Gln Ala Glu Asp Ala Arg Lys Ala Ile Asp Ala Ala Glu
      40                  45                  50

cgt gca caa cca gaa tgg gaa gcg ttg cct gct att gaa cgc gcc agt   246
Arg Ala Gln Pro Glu Trp Glu Ala Leu Pro Ala Ile Glu Arg Ala Ser
 55                  60                  65                  70

tgg ttg cgc aaa atc tcc gcc ggg atc cgc gaa cgc gcc agt gaa atc   294
Trp Leu Arg Lys Ile Ser Ala Gly Ile Arg Glu Arg Ala Ser Glu Ile
                 75                  80                  85

agt gcg ctg att gtt gaa gaa ggg ggc aag atc cag cag ctg gct gaa   342
Ser Ala Leu Ile Val Glu Glu Gly Gly Lys Ile Gln Gln Leu Ala Glu
            90                  95                 100

gtc gaa gtg gct ttt act gcc gac tat atc gat tac atg gcg gag tgg   390
Val Glu Val Ala Phe Thr Ala Asp Tyr Ile Asp Tyr Met Ala Glu Trp
        105                 110                 115

gca cgg cgt tac gag ggc gag att att caa agc gat cgt cca gga gaa   438
Ala Arg Arg Tyr Glu Gly Glu Ile Ile Gln Ser Asp Arg Pro Gly Glu
    120                 125                 130

aat att ctt ttg ttt aaa cgt gcg ctt ggt gtg act acc ggc att ctg   486
Asn Ile Leu Leu Phe Lys Arg Ala Leu Gly Val Thr Thr Gly Ile Leu
135                 140                 145                 150

ccg tgg aac ttc ccg ttc ttc ctc att gcc cgc aaa atg gct ccc gct   534
Pro Trp Asn Phe Pro Phe Phe Leu Ile Ala Arg Lys Met Ala Pro Ala
                155                 160                 165
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ctt ttg acc ggt aat acc atc gtc att aaa cct agt gaa ttt acg aca   582
Leu Leu Thr Gly Asn Thr Ile Val Ile Lys Pro Ser Glu Phe Thr Thr
            170                 175                 180

aac aat gcg att gca ttc gcc aaa atc gtc gat gaa ata ggc ctt ccg   630
Asn Asn Ala Ile Ala Phe Ala Lys Ile Val Asp Glu Ile Gly Leu Pro
        185                 190                 195

cgc ggc gtg ttt aac ctt gta ctg ggg cgt ggt gaa acc gtt ggg caa   678
Arg Gly Val Phe Asn Leu Val Leu Gly Arg Gly Glu Thr Val Gly Gln
    200                 205                 210

gaa ctg gcg ggt aac cca aag gtc gca atg gtc agt atg aca ggc agc   726
Glu Leu Ala Gly Asn Pro Lys Val Ala Met Val Ser Met Thr Gly Ser
215                 220                 225                 230

gtc tct gca ggt gag aag atc atg gcg act gcg gcg aaa aac atc acc   774
Val Ser Ala Gly Glu Lys Ile Met Ala Thr Ala Ala Lys Asn Ile Thr
                235                 240                 245

aaa gtg tgt ctg gaa ttg ggg ggt aaa gca cca gct atc gta atg gac   822
Lys Val Cys Leu Glu Leu Gly Gly Lys Ala Pro Ala Ile Val Met Asp
            250                 255                 260

gat gcc gat ctt gaa ctg gca gtc aaa gcc atc gtt gat tca cgc gtc   870
Asp Ala Asp Leu Glu Leu Ala Val Lys Ala Ile Val Asp Ser Arg Val
        265                 270                 275

att aat agt ggg caa gtg tgt aac tgt gca gaa cgt gtt tat gta cag   918
Ile Asn Ser Gly Gln Val Cys Asn Cys Ala Glu Arg Val Tyr Val Gln
    280                 285                 290

aaa ggc att tat gat cag ttc gtc aat cgg ctg ggt gaa gcg atg cag   966
Lys Gly Ile Tyr Asp Gln Phe Val Asn Arg Leu Gly Glu Ala Met Gln
295                 300                 305                 310
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gcg gtt caa ttt ggt aac ccc gct gaa cgc aac gac att gcg atg ggg   1014
Ala Val Gln Phe Gly Asn Pro Ala Glu Arg Asn Asp Ile Ala Met Gly
                315                 320                 325

ccg ttg att aac gcc gcg gcg ctg gaa agg gtc gag caa aaa gtg gcg   1062
Pro Leu Ile Asn Ala Ala Ala Leu Glu Arg Val Glu Gln Lys Val Ala
            330                 335                 340

cgc gca gta gaa gaa ggg gcg aga gtg gcg ttc ggt ggc aaa gcg gta   1110
Arg Ala Val Glu Glu Gly Ala Arg Val Ala Phe Gly Gly Lys Ala Val
        345                 350                 355

gag ggg aaa gga tat tat tat ccg ccg aca ttg ctg ctg gat gtt cgc   1158
Glu Gly Lys Gly Tyr Tyr Tyr Pro Pro Thr Leu Leu Leu Asp Val Arg
    360                 365                 370

cag gaa atg tcg att atg cat gag gaa acc ttt ggc ccg gtg ctg cca   1206
Gln Glu Met Ser Ile Met His Glu Glu Thr Phe Gly Pro Val Leu Pro
375                 380                 385                 390

gtt gtc gca ttt gac acg ctg gaa gat gct atc tca atg gct aat gac   1254
Val Val Ala Phe Asp Thr Leu Glu Asp Ala Ile Ser Met Ala Asn Asp
                395                 400                 405

agt gat tac ggc ctg acc tca tca atc tat acc caa aat ctg aac gtc   1302
Ser Asp Tyr Gly Leu Thr Ser Ser Ile Tyr Thr Gln Asn Leu Asn Val
            410                 415                 420

gcg atg aaa gcc att aaa ggg ctg aag ttt ggt gaa act tac atc aac   1350
Ala Met Lys Ala Ile Lys Gly Leu Lys Phe Gly Glu Thr Tyr Ile Asn
        425                 430                 435

cgt gaa aac ttc gaa gct atg caa ggc ttc cac gcc gga tgg cgt aaa   1398
Arg Glu Asn Phe Glu Ala Met Gln Gly Phe His Ala Gly Trp Arg Lys
    440                 445                 450
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tcc ggt att ggc ggc gca gat ggt aaa cat ggc ttg cat gga tat ctg 1446
Ser Gly Ile Gly Gly Ala Asp Gly Lys His Gly Leu His Gly Tyr Leu
455 460 465 470

cag acc cag gtg gtt tat tta cag tct taagagctcg aattcccgtc 1493
Gln Thr Gln Val Val Tyr Leu Gln Ser
475

gacggctcta gactcgagcg 1513

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Ala Val Ile Ser Arg Ile Pro Asp Gly Gln Ala Glu Asp Ala Arg Lys
35 40 45

Ala Ile Asp Ala Ala Glu Arg Ala Gln Pro Glu Trp Glu Ala Leu Pro
50 55 60

Ala Ile Glu Arg Ala Ser Trp Leu Arg Lys Ile Ser Ala Gly Ile Arg
65 70 75 80

Glu Arg Ala Ser Glu Ile Ser Ala Leu Ile Val Glu Glu Gly Gly Lys
85 90 95

Ile Gln Gln Leu Ala Glu Val Glu Val Ala Phe Thr Ala Asp Tyr Ile
100 105 110

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Asp Tyr Met Ala Glu Trp Ala Arg Arg Tyr Glu Gly Glu Ile Ile Gln
        115                 120                 125

Ser Asp Arg Pro Gly Glu Asn Ile Leu Leu Phe Lys Arg Ala Leu Gly
    130                 135                 140

Val Thr Thr Gly Ile Leu Pro Trp Asn Phe Pro Phe Phe Leu Ile Ala
145                 150                 155                 160

Arg Lys Met Ala Pro Ala Leu Leu Thr Gly Asn Thr Ile Val Ile Lys
                165                 170                 175

Pro Ser Glu Phe Thr Thr Asn Asn Ala Ile Ala Phe Ala Lys Ile Val
            180                 185                 190

Asp Glu Ile Gly Leu Pro Arg Gly Val Phe Asn Leu Val Leu Gly Arg
        195                 200                 205

Gly Glu Thr Val Gly Gln Glu Leu Ala Gly Asn Pro Lys Val Ala Met
    210                 215                 220

Val Ser Met Thr Gly Ser Val Ser Ala Gly Glu Lys Ile Met Ala Thr
225                 230                 235                 240

Ala Ala Lys Asn Ile Thr Lys Val Cys Leu Glu Leu Gly Gly Lys Ala
                245                 250                 255

Pro Ala Ile Val Met Asp Asp Ala Asp Leu Glu Leu Ala Val Lys Ala
            260                 265                 270

Ile Val Asp Ser Arg Val Ile Asn Ser Gly Gln Val Cys Asn Cys Ala
        275                 280                 285

Glu Arg Val Tyr Val Gln Lys Gly Ile Tyr Asp Gln Phe Val Asn Arg
    290                 295                 300
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Leu Gly Glu Ala Met Gln Ala Val Gln Phe Gly Asn Pro Ala Glu Arg
305 310 315 320

Asn Asp Ile Ala Met Gly Pro Leu Ile Asn Ala Ala Ala Leu Glu Arg
325 330 335

Val Glu Gln Lys Val Ala Arg Ala Val Glu Glu Gly Ala Arg Val Ala
340 345 350

Phe Gly Gly Lys Ala Val Glu Gly Lys Gly Tyr Tyr Tyr Pro Pro Thr
355 360 365

Leu Leu Leu Asp Val Arg Gln Glu Met Ser Ile Met His Glu Glu Thr
370 375 380

Phe Gly Pro Val Leu Pro Val Val Ala Phe Asp Thr Leu Glu Asp Ala
385 390 395 400

Ile Ser Met Ala Asn Asp Ser Asp Tyr Gly Leu Thr Ser Ser Ile Tyr
405 410 415

Thr Gln Asn Leu Asn Val Ala Met Lys Ala Ile Lys Gly Leu Lys Phe
420 425 430

Gly Glu Thr Tyr Ile Asn Arg Glu Asn Phe Glu Ala Met Gln Gly Phe
435 440 445

His Ala Gly Trp Arg Lys Ser Gly Ile Gly Gly Ala Asp Gly Lys His
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Gly Leu His Gly Tyr Leu Gln Thr Gln Val Val Tyr Leu Gln Ser
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aag ccc ggc gag tat ggt ttc ccc ctc aag tta aaa gcc cgc tat gac 99
Lys Pro Gly Glu Tyr Gly Phe Pro Leu Lys Leu Lys Ala Arg Tyr Asp
15 20 25

aac ttt att ggc ggc gaa tgg gta gcc cct gcc gac ggc gag tat tac 147
Asn Phe Ile Gly Gly Glu Trp Val Ala Pro Ala Asp Gly Glu Tyr Tyr
30 35 40

cag aat ctg acg ccg gtg acc ggg cag ctg ctg tgc gaa gtg gcg tct 195
Gln Asn Leu Thr Pro Val Thr Gly Gln Leu Leu Cys Glu Val Ala Ser
45 50 55

tcg ggc aaa cga gac atc gat ctg gcg ctg gat gct gcg cac aaa gtg 243
Ser Gly Lys Arg Asp Ile Asp Leu Ala Leu Asp Ala Ala His Lys Val
60 65 70

aaa gat aaa tgg gcg cac acc tcg gtg cag gat cgt gcg gcg att ctg 291
Lys Asp Lys Trp Ala His Thr Ser Val Gln Asp Arg Ala Ala Ile Leu
75 80 85 90

ttt aag att gcc gat cga atg gaa caa aac ctc gag ctg tta gcg aca 339
Phe Lys Ile Ala Asp Arg Met Glu Gln Asn Leu Glu Leu Leu Ala Thr
95 100 105

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gct gaa acc tgg gat aac ggc aaa ccc att cgc gaa acc agt gct gcg   387
Ala Glu Thr Trp Asp Asn Gly Lys Pro Ile Arg Glu Thr Ser Ala Ala
            110                 115                 120

gat gta ccg ctg gcg att gac cat ttc cgc tat ttc gcc tcg tgt att   435
Asp Val Pro Leu Ala Ile Asp His Phe Arg Tyr Phe Ala Ser Cys Ile
        125                 130                 135

cgg gcg cag gaa ggt ggg atc agt gaa gtt gat agc gaa acc gtg gcc   483
Arg Ala Gln Glu Gly Gly Ile Ser Glu Val Asp Ser Glu Thr Val Ala
    140                 145                 150

tat cat ttc cat gaa ccg tta ggc gtg gtg ggg cag att atc ccg tgg   531
Tyr His Phe His Glu Pro Leu Gly Val Val Gly Gln Ile Ile Pro Trp
155                 160                 165                 170

aac ttc ccg ctg ctg atg gcg agc tgg aaa atg gct ccc gcg ctg gcg   579
Asn Phe Pro Leu Leu Met Ala Ser Trp Lys Met Ala Pro Ala Leu Ala
                175                 180                 185

gcg ggc aac tgt gtg gtg ctg aaa ccc gca cgt ctt acc ccg ctt tct   627
Ala Gly Asn Cys Val Val Leu Lys Pro Ala Arg Leu Thr Pro Leu Ser
            190                 195                 200

gta ctg ctg cta atg gaa att gtc ggt gat tta ctg ccg ccg ggc gtg   675
Val Leu Leu Leu Met Glu Ile Val Gly Asp Leu Leu Pro Pro Gly Val
        205                 210                 215

gtg aac gtg gtc aat ggc gca ggt ggg gta att ggc gaa tat ctg gcg   723
Val Asn Val Val Asn Gly Ala Gly Gly Val Ile Gly Glu Tyr Leu Ala
    220                 225                 230

acc tcg aaa cgc atc gcc aaa gtg gcg ttt acc ggc tca acg gaa gtg   771
Thr Ser Lys Arg Ile Ala Lys Val Ala Phe Thr Gly Ser Thr Glu Val
235                 240                 245                 250
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ggc caa caa att atg caa tac gca acg caa aac att att ccg gtg acg   819
Gly Gln Gln Ile Met Gln Tyr Ala Thr Gln Asn Ile Ile Pro Val Thr
                255                 260                 265

ctg gag ttg ggc ggt aag tcg cca aat atc gtc ttt gct gat gtg atg   867
Leu Glu Leu Gly Gly Lys Ser Pro Asn Ile Val Phe Ala Asp Val Met
            270                 275                 280

gat gaa gaa gat gcc ttt ttc gat aaa gcg ctg gaa ggc ttt gca ctg   915
Asp Glu Glu Asp Ala Phe Phe Asp Lys Ala Leu Glu Gly Phe Ala Leu
        285                 290                 295

ttt gcc ttt aac cag ggc gaa gtt tgc acc tgt ccg agt cgt gct tta   963
Phe Ala Phe Asn Gln Gly Glu Val Cys Thr Cys Pro Ser Arg Ala Leu
    300                 305                 310

gtg cag gaa tct atc tac gaa cgc ttt atg gaa cgc gcc atc cgc cgt  1011
Val Gln Glu Ser Ile Tyr Glu Arg Phe Met Glu Arg Ala Ile Arg Arg
315                 320                 325                 330

gtc gaa agc att cgt agc ggt aac ccg ctc gac agc gtg acg caa atg  1059
Val Glu Ser Ile Arg Ser Gly Asn Pro Leu Asp Ser Val Thr Gln Met
                335                 340                 345

ggc gcg cag gtt tct cac ggg caa ctg gaa acc atc ctc aac tac att  1107
Gly Ala Gln Val Ser His Gly Gln Leu Glu Thr Ile Leu Asn Tyr Ile
            350                 355                 360

gat atc ggt aaa aaa gag ggc gct gac gtg ctc aca ggc ggg cgg cgc  1155
Asp Ile Gly Lys Lys Glu Gly Ala Asp Val Leu Thr Gly Gly Arg Arg
        365                 370                 375

aag ctg ctg gaa ggt gaa ctg aaa gac ggc tac tac ctc gaa ccg acg  1203
Lys Leu Leu Glu Gly Glu Leu Lys Asp Gly Tyr Tyr Leu Glu Pro Thr
    380                 385                 390
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att ctg ttt ggt cag aac aat atg cgg gtg ttc cag gag gag att ttt   1251
Ile Leu Phe Gly Gln Asn Asn Met Arg Val Phe Gln Glu Glu Ile Phe
395                 400                 405                 410

ggc ccg gtg ctg gcg gtg acc acc ttc aaa acg atg gaa gaa gcg ctg   1299
Gly Pro Val Leu Ala Val Thr Thr Phe Lys Thr Met Glu Glu Ala Leu
                415                 420                 425

gag ctg gcg aac gat acg caa tat ggc ctg ggc gcg ggc gtc tgg agc   1347
Glu Leu Ala Asn Asp Thr Gln Tyr Gly Leu Gly Ala Gly Val Trp Ser
            430                 435                 440

cgc aac ggt aat ctg gcc tat aag atg ggg cgc ggc ata cag gct ggg   1395
Arg Asn Gly Asn Leu Ala Tyr Lys Met Gly Arg Gly Ile Gln Ala Gly
        445                 450                 455

cgc gtg tgg acc aac tgt tat cac gct tac ccg gca cat gcg gcg ttt   1443
Arg Val Trp Thr Asn Cys Tyr His Ala Tyr Pro Ala His Ala Ala Phe
    460                 465                 470

ggt ggc tac aaa caa tca ggt atc ggt cgc gaa acc cac aag atg atg   1491
Gly Gly Tyr Lys Gln Ser Gly Ile Gly Arg Glu Thr His Lys Met Met
475                 480                 485                 490

ctg gag cat tac cag caa acc aag tgc ctg ctg gtg agc tac tcg gat   1539
Leu Glu His Tyr Gln Gln Thr Lys Cys Leu Leu Val Ser Tyr Ser Asp
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aaa ccg ttg ggg ctg ttc taagagctcg aattcgc                       1574
Lys Pro Leu Gly Leu Phe
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Phe Pro Leu Lys Leu Lys Ala Arg Tyr Asp Asn Phe Ile Gly Gly Glu
             20                  25                  30

Trp Val Ala Pro Ala Asp Gly Glu Tyr Tyr Gln Asn Leu Thr Pro Val
          35                  40                  45

Thr Gly Gln Leu Leu Cys Glu Val Ala Ser Ser Gly Lys Arg Asp Ile
      50                  55                  60

Asp Leu Ala Leu Asp Ala Ala His Lys Val Lys Asp Lys Trp Ala His
  65                  70                  75                  80

Thr Ser Val Gln Asp Arg Ala Ala Ile Leu Phe Lys Ile Ala Asp Arg
                  85                  90                  95

Met Glu Gln Asn Leu Glu Leu Leu Ala Thr Ala Glu Thr Trp Asp Asn
             100                 105                 110

Gly Lys Pro Ile Arg Glu Thr Ser Ala Ala Asp Val Pro Leu Ala Ile
          115                 120                 125

Asp His Phe Arg Tyr Phe Ala Ser Cys Ile Arg Ala Gln Glu Gly Gly
      130                 135                 140

Ile Ser Glu Val Asp Ser Glu Thr Val Ala Tyr His Phe His Glu Pro
  145                 150                 155                 160
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Leu Gly Val Val Gly Gln Ile Ile Pro Trp Asn Phe Pro Leu Leu Met
                165                 170                 175

Ala Ser Trp Lys Met Ala Pro Ala Leu Ala Ala Gly Asn Cys Val Val
            180                 185                 190

Leu Lys Pro Ala Arg Leu Thr Pro Leu Ser Val Leu Leu Leu Met Glu
        195                 200                 205

Ile Val Gly Asp Leu Leu Pro Pro Gly Val Val Asn Val Val Asn Gly
    210                 215                 220

Ala Gly Gly Val Ile Gly Glu Tyr Leu Ala Thr Ser Lys Arg Ile Ala
225                 230                 235                 240

Lys Val Ala Phe Thr Gly Ser Thr Glu Val Gly Gln Gln Ile Met Gln
                245                 250                 255

Tyr Ala Thr Gln Asn Ile Ile Pro Val Thr Leu Glu Leu Gly Gly Lys
            260                 265                 270

Ser Pro Asn Ile Val Phe Ala Asp Val Met Asp Glu Glu Asp Ala Phe
        275                 280                 285

Phe Asp Lys Ala Leu Glu Gly Phe Ala Leu Phe Ala Phe Asn Gln Gly
    290                 295                 300

Glu Val Cys Thr Cys Pro Ser Arg Ala Leu Val Gln Glu Ser Ile Tyr
305                 310                 315                 320

Glu Arg Phe Met Glu Arg Ala Ile Arg Arg Val Glu Ser Ile Arg Ser
                325                 330                 335

Gly Asn Pro Leu Asp Ser Val Thr Gln Met Gly Ala Gln Val Ser His
            340                 345                 350
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Gly Gln Leu Glu Thr Ile Leu Asn Tyr Ile Asp Ile Gly Lys Lys Glu
355 360 365

Gly Ala Asp Val Leu Thr Gly Gly Arg Arg Lys Leu Leu Glu Gly Glu
370 375 380

Leu Lys Asp Gly Tyr Tyr Leu Glu Pro Thr Ile Leu Phe Gly Gln Asn
385 390 395 400

Asn Met Arg Val Phe Gln Glu Glu Ile Phe Gly Pro Val Leu Ala Val
405 410 415

Thr Thr Phe Lys Thr Met Glu Glu Ala Leu Glu Leu Ala Asn Asp Thr
420 425 430

Gln Tyr Gly Leu Gly Ala Gly Val Trp Ser Arg Asn Gly Asn Leu Ala
435 440 445

Tyr Lys Met Gly Arg Gly Ile Gln Ala Gly Arg Val Trp Thr Asn Cys
450 455 460

Tyr His Ala Tyr Pro Ala His Ala Ala Phe Gly Gly Tyr Lys Gln Ser
465 470 475 480

Gly Ile Gly Arg Glu Thr His Lys Met Met Leu Glu His Tyr Gln Gln
485 490 495

Thr Lys Cys Leu Leu Val Ser Tyr Ser Asp Lys Pro Leu Gly Leu Phe
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     50                  55                  60

Ser Asp Val Ser Arg Ile Tyr Leu Asn Glu Ala Ala Pro Val Ile Gly
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Asp Val Ala Met Glu Thr Ile Thr Glu Thr Ile Ile Thr Glu Ser Thr
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Met Ile Gly His Asn Pro Gln Thr Pro Gly Gly Val Gly Val Gly Val
            100                 105                 110

Gly Thr Thr Ile Ala Leu Gly Arg Leu Ala Thr Leu Pro Ala Ala Gln
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Tyr Ala Glu Gly Trp Ile Val Leu Ile Asp Asp Ala Val Asp Phe Leu
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Val Ala Ala Ile Leu Lys Lys Asp Asp Gly Val Leu Val Asn Asn Arg
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Gln Ser Arg Val Ile Pro Ala Gly Asn Leu Tyr Ile Ser Gly Glu Lys
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Thr His Ala Gly Gly Met Leu Glu Arg Val Arg Lys Val Met Ala Ser
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Thr Glu Val Val Val Gly Gly Val Glu Ala Asn Met Ala Ile Ala Gly
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Ser Phe Met Ala Trp Asp Ala Ala Asn Leu Ser Gly Ser Gly Ile Gly
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Ile Gly Ile Gln Ser Lys Gly Thr Thr Val Ile His Gln Arg Asp Leu
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Leu Pro Leu Ser Asn Leu Glu Leu Phe Ser Gln Ala Pro Leu Leu Thr
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Leu Glu Thr Tyr Arg Gln Ile Gly Lys Asn Ala Ala Arg Tyr Ala Arg
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Lys Glu Ser Pro Ser Pro Val Pro Val Val Asn Asp Gln Met Val Arg
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Pro Lys Phe Met Ala Lys Ala Ala Leu Phe His Ile Lys Glu Thr Lys
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Ile Val Glu Leu Asp Gly Lys Arg Arg Asp Gln Phe Asp Met Ile Asp
     50                  55                  60

Arg Phe Ile Ala Asp Tyr Ala Ile Asn Val Glu Arg Thr Glu Gln Ala
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Met Arg Leu Glu Ala Val Glu Ile Ala Arg Met Leu Val Asp Ile His
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Val Ser Arg Glu Glu Ile Ile Ala Ile Thr Thr Ala Ile Thr Pro Ala
            100                 105                 110

Lys Ala Val Glu Val Met Ala Gln Met Asn Val Val Glu Met Met Met
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Ala Leu Gln Lys Met Arg Ala Arg Arg Thr Pro Ser Asn Gln Cys His
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Cys Gly Arg Pro Gly Val Leu Thr Gln Cys Ser Val Glu Glu Ala Thr
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QBMAD\223318

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US00/23878

A. CLASSIFICATION OF SUBJECT MATTER

IPC(7) :C12P 7/22; C12N 9/02, 9/14, 1/20, 15/00
US CL :435/155, 189, 195, 252.3, 320.1

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 435/155, 189, 195, 252.3, 320.1

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

West; STN files included medline, caplus, uspatfull, embase, sciscarch, biosis and biotechds

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	TANG et al. Immunochemical Properties of NAD+ - Linked Glycerol Dehydrogenases from Escherichia coli and Klebsiella pneumoniae. Journal of Bacteriology. December 1982, Vol. 152, No. 3, pages 1169-1174, see the entire article.	1-12
A	BARBIRATO et al. Anaerobic pathways of glycerol dissimilation by Enterobacter agglomerans CNCM 1210: limitations and regulations. Microbiology. 1997, Vol. 143, No. 7, pages 2423-2432.	1-12

☐ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

*	Special categories of cited documents:	"T"	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"A"	document defining the general state of the art which is not considered to be of particular relevance	"X"	document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"E"	earlier document published on or after the international filing date	"Y"	document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"L"	document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	"&"	document member of the same patent family
"O"	document referring to an oral disclosure, use, exhibition or other means		
"P"	document published prior to the international filing date but later than the priority date claimed		

Date of the actual completion of the international search 16 OCTOBER 2000	Date of mailing of the international search report 30 NOV 2000
Name and mailing address of the ISA/US Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231 Facsimile No. (703) 305-3230	Authorized officer TEKCHAND SAIDHA Telephone No. (703) 308-0196

Form PCT/ISA/210 (second sheet) (July 1998)★